The Question of Functional Homology of Hatschek's Pit of Amphioxus (Branchiostoma belcheri) and the Vertebrate Adenohypophysis

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The Question of Functional Homology of Hatschek's Pit of Amphioxus (*Branchiostoma belcheri*) and the Vertebrate Adenohypophysis

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ABSTRACT—Using antibodies to the beta subunit of human luteinizing hormone (hLHβ) and human chorionic gonadotropin, immunocytochemical evidence was obtained for gonadotropin activity in Hatschek's pit of amphioxus, *Branchiostoma belcheri*. This confirms the claim by C. Y. Chang *et al.* [1, 2] of vertebrate-like gonadotropin in this structure, an open groove in the dorsal part of the oral cavity. If this evidence is accepted at face value, a scenario can be constructed for the evolutionary pattern of the vertebrate adenohypophysis from the protochordate Hatschek's pit (cephalochordates) or neural gland (ascidians). Both of these structures are open to water currents in the mouth cavity. Thus, they may be able to sample thermal, chemical or pheromonal seasonally cycling clues and by gonadotropic stimulation, synchronize reproductive activity with such seasonal clues. Additional support for the idea that the early vertebrate adenohypophysis was a chemoreceptive organ comes from the fact that in cyclostomes and elasmobranchs it develops as part of the same epithelial layer and is directly contiguous with the olfactory organ. Advancement from the protochordate to vertebrate type of reproductive control involves the eventual use of sense organs and the nervous system to sample environmental changes, and the linkage of adenohypophysial function to central nervous control. The adenohypophysis then can be closed off from the mouth and direct environmental contact.

INTRODUCTION

Considerable interest by comparative endocrinologists was ignited by the reports by C. Y. Chang *et al.* [1, 2] that in amphioxus immunoreactive responses in Hatschek's pit to antibodies to mammalian gonadotropins could be obtained. Evidence reported from Chang's laboratory indicated also that administration of ovine luteinizing hormone (LH) and prolactin to amphioxus increased the whole-body concentrations of sex steroids [2]. Furthermore, immunoreactive gonadotropin-releasing hormone (GnRH) and thyrotropin-releasing hormone (TRH) were found in Hatschek's pit, and saturable receptor activity for mammalian LH/human chorionic gonadotropin (hCG) and GnRH could be measured in gonads of amphioxus [2].

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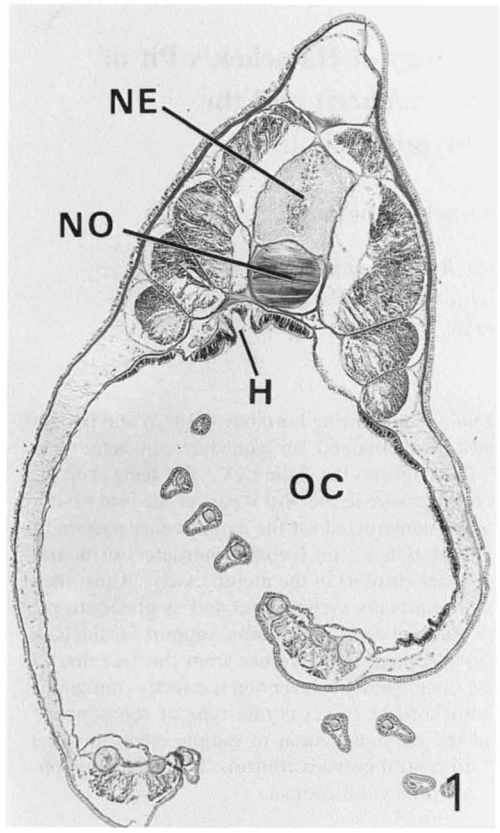
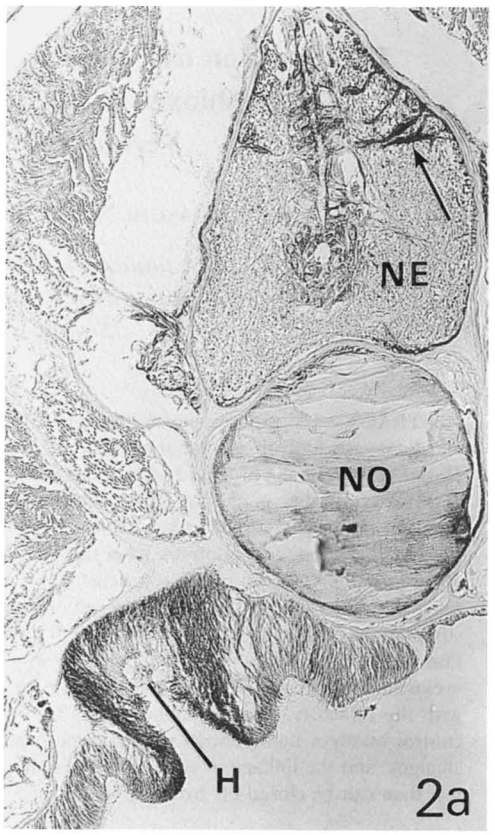
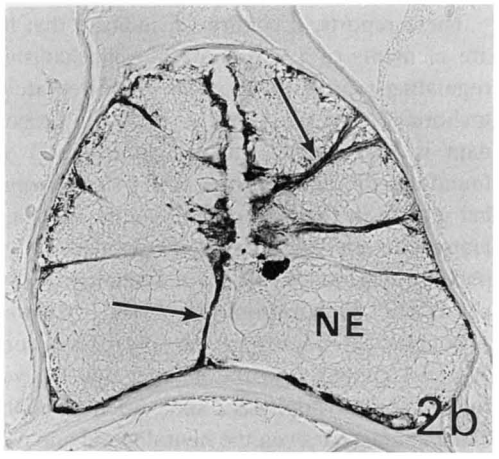
These reports, if confirmed, indicate that there are elements of a vertebrate-like mechanism for regulating reproduction in this prevertebrate protochordate. A puzzling aspect of Chang's reported data is that GnRH and gonadotropin(s) were found together in Hatschek's pit, a shallow epithelial groove in the roof of the oral cavity (Fig. 1). Hatschek's pit has long been regarded, on morphological grounds, to be a homologue of the vertebrate adenohypophysis [3–6]. However, although it extends toward the dorsal nerve cord, it does not contact it in the manner that the vertebrate neurohypophysis and adenohypophysis make contact, or even the neural gland and neural ganglion of ascidians.

Chang's reports stimulated efforts in other laboratories to confirm them. Among them, Fang and Wang [7] found that administration of homogenates of *Branchiostoma belcheri* Hatschek's pits stimulates testicular spermiation in young toads. Sahlin [8], in an immunohistoche-







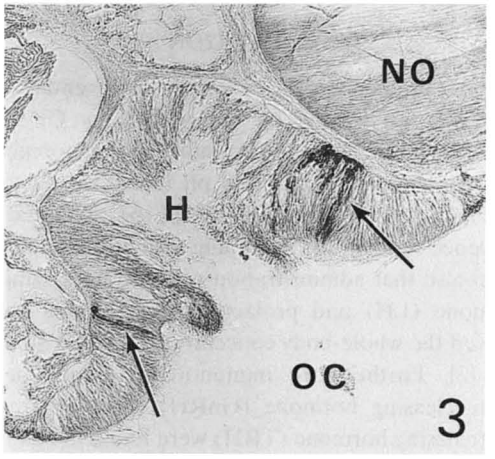


FIG. 1. Transverse section through Hatschek's pit (H) showing topographic relations of Hatschek's pit, notochord (NO), and nerve cord (NE). OC, oral cavity. Hematoxylin and eosin stain. ×75.

FIG. 2. Anti-substance P immunostain. **a**, Transverse section through Hatschek's pit (H). In the Hatschek's pit, all the material reactive as substance P are evenly distributed among the cells of the pit, with possibly greater intensity in the lateral areas, whereas the nerve cord contains some darkly stained cells in the dorsal region. **b**, Transverse section at the level of the oesophagus and the middle part of the body, showing substance P-positive immunoreaction in cells of the nerve cord (NE, arrows). NO, notochord. a, ×240; b, ×310.

FIG. 3. Transverse section through Hatschek's pit (H) stained with anti-Met-enkephalin. Immunostaining is more restricted to the lateral margins of the pit, specifically limited to particular cells. NO, notochord; OC, oral cavity. ×270.

mical study using *Branchiostoma lanceolatum,* found no response to antibodies to a variety of vertebrate neurohypophysial and hypophysial hormones (including gonadotropins) in Hatschek's pit. However, she observed a clear reaction to an antibody to the C-terminal portion of CCK.

Because of the importance of the evolutionary implications of Chang's data, and because of the failure by others to confirm them until now, we have undertaken an immunohistochemical study of Hatschek's pit, using the same species, *Branchiostoma belcheri.*

MATERIALS AND METHODS

Animals

Specimens of *Branchiostoma belcheri* were collected during the month of April, 1987, at Tsuyazaki, a village on the northwest shore of Kyushu Island, Japan. They were collected in a large single sample of sand brought up from a depth of about 20 m by dredge. They measured 2.5 to 5.9 cm and weighed 0.03 to 0.49 g. According to Yamaguchi and Kikuchi [9], the amphioxi from various collection sites around Kyushu Island vary slightly in myotome number, but all are classified *Branchiostoma belcheri,* or *Branchiostoma belcheri var. tsingtaoense,* or intermediate forms between these. Chang et al. [1] stated that they used both of these forms in their experiments.

Treatment

The heads were removed and immersed in Bouin-Hollande sublimate for about 12 hr. They were dehydrated through a series of increasing concentrations of ethanol. After 90% ethanol, the tissue were washed in a solution containing iodine-potassium iodide in 90% ethanol for 24 hr to remove deposited mercuric chloride. Tissues were embedded in Paraplast, and serial sections of 6 μm were mounted on glass slides. Immunocytochemical staining was performed with a Vectastain ABC (avidin-biotin peroxidase complex) kit using a variety of polyclonal antibodies to hypothalamic, hypophysial, pancreatic and gut hormones from a number of vertebrates (Table 1). The staining procedures have been described previously [10]. Specificity of the reactions was checked by replacing the primary antibodies with normal sera or by using primary antibodies that were previously absorbed with corresponding antigens.

RESULTS AND DISCUSSION

Of the 28 antibodies tested, two yielded clear stains of cells in Hatschek's pit (substance P and met-enkephalin; Table 1). Two yielded weaker results (hLHβ and hCG; Table 1). Preabsorption of any of these four antibodies by the primary antigens blocked the staining reaction, so that in this sense, at least, the results may be considered specific reactions to the antibodies. The strength of the reaction with substance P and met-enkephalin antibodies should indicate a relatively close molecular similarity of the stained materials to the primary antigens against which the antibodies were produced. The weakness of the reaction to the human gonadotropin antibodies argues that molecules bearing limited structural relation to hLHβ and hCG exist in Hatschek's pit. Thus, our results confirm the report by Chang *et al.* [1, 2] of a vertebrate-like gonadotropin in Hatschek's pit. However, we could not confirm the report of the presence of immunoreactive GnRH in Hatschek's pit.

Substance P, a neurotransmitter in the central nervous system, was quite generally distributed in Hatschek's pit, particularly in the cells of the lateral portions (Fig. 2). It also was seen in cells of the nerve cord along its entire length (Figs. 2a and b). Met-enkephalin immunoreactivity was clearly limited to cells near the lateral margins of the pit (Fig. 3).

The hLHβ positive cells were consistently in the deep portion of the pit, adjacent to the notochord (Fig. 4a). The anti-hCG antibody likewise reacted with cells in the deeper parts of Hatschek's pit (Fig. 4b), but not as consistently as with the hLHβ antibody. The relative weakness of the stain raises doubts concerning specificity of the gonadotropin antibody-labeling. These doubts are reinforced by the lack of immunostaining following use of two antibodies to two fish (silver carp and salmon) gonadotropins. However, arguing in favor of the significance and specificity of this gonadotropin

TABLE 1. List of antibodies used and immunoreactions to them in Hatschek's pit

Antibodies* to		Obtained from	Immunoreactivity response	Optimum dilution	References
Hypothalamic hormones	mammalian-GnRH	Miles-Yeda Co.	−	1000	26
	lamprey-GnRH	J. A. King	−		
	SRIH-14	Polysciences Co.	−	500	36
	AVP	Raised in laboratory	−	400	36
Pituitary hormones I	human-LHβ	NIAMDD	+	1000	
	human-FSHβ	NIAMDD	−	1000	
	human-TSHβ	NIAMDD	−	500	
	human-CG	Raised in laboratory	+/−	1000	
	salmon-GTH	M. Kobayashi	−	500	37
	silver carp-GTH	M. Kobayashi	−	300	38
Pituitary hormones II	human-PRL	NIAMDD	−	1000	
	human-GH	NIAMDD	−	1000	
	porcine-ACTH	Raised in laboratory	−	400	39
	α-MSH	B. Baker	−	300	39
	salmon-PRL	H. Kawauchi	−	4000	40
	salmon-GH	H. Kawauchi	−	2000	41
Pancreatic hormones	human-insulin	E. Plisetskaya	−	400	
	human-glucagon	Raised in laboratory	−	5000	42
	porcine-PP	Funakoshi Co.	−	500	
	salmon-insulin	E. Plisetskaya	−	2000	10
	salmon-glucagon	E. Plisetskaya	−	600	10
	salmon-SRIH-25	E. Plisetskaya	−	1200	10
Brain-gut peptide	CCK-8	Funakoshi Co.	−	400	
	CCK-27	N. Yanaibara	−	600	
	porcine-VIP	N. Yanaihara	−	500	
	Substance P	Raised in laboratory	++	800	43
	Neurotensin	N. Yanaihara	−	1000	
	Met-enkephalin	Raised in Laboratory	++	400	39

* Abbreviations: ACTH, adrenocorticotropin; AVP, arginine vasopressin; CCK-8, cholecystokinin octapeptide; CG, chorionic gonadotropin; FSHβ, beta subunit of follicle-stimulating hormone; GH, growth hormone; GnRH, gonadotropin-releasing hormone; GTH, gonadotropic hormone; LHβ, beta subunit of luteinizing hormone; α-MSH, alpha-melanocyte-stimulating hormone; NIAMDD, U.S. National Institute of Health; PP, pancreatic polypeptide; PRL, prolactin; SRIH, somatostatin; TSHβ, beta subunit of thyrotropic hormone; VIP, vasoactive intestinal polypeptide.

immunostain are the following: (1) both LH and hCG are luteinizing hormones and may be presumed to share antigenic determinants; (2) hFSHβ antibody yielded no stain; (3) preabsorption of the two positive antibodies with their respective antigens blocked the immunostaining.

Concerning the significance of the clear reactions for substance P and met-enkephalin we can say little at this time. These substances have been reported in analogous structures, the neural gland and ganglion of ascidians [11, 12], but their physiological significance requires additional study to define.

Concerning the weak responses that we found for LH and LH-like gonadotropin, it is tempting to speculate, particularly since Chang and associates

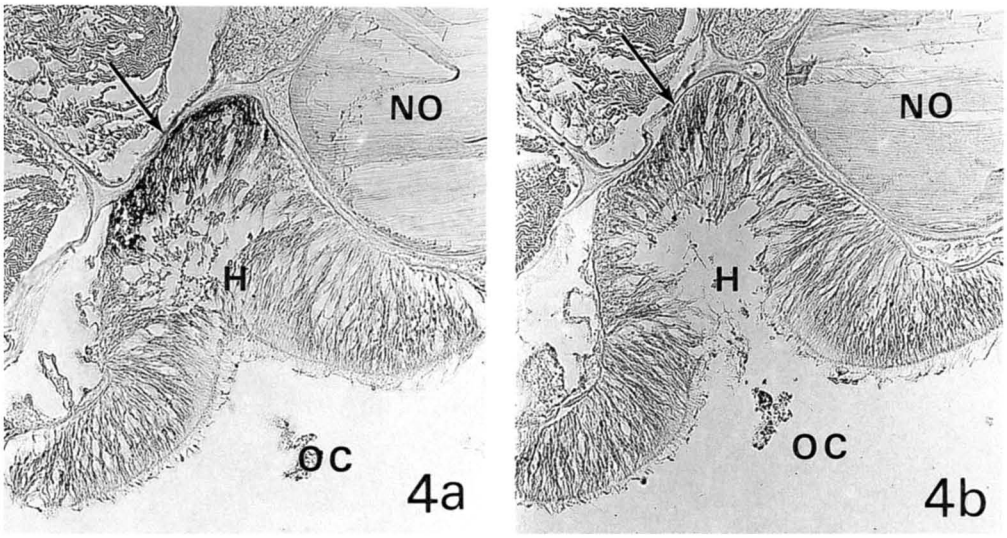


FIG. 4. Two adjacent, but not successive, sections stained with anti-hLHβ (**a**) and anti-hCG (**b**). Cells in the deepest part of Hatschek's pit were stained consistently with anti-hLHβ (arrow in **a**), whereas such cells (arrow in **b**) were stained weekly with anti-hCG. OC, oral cavity. **a** and **b**, $\times 270$.

have proposed that they play a role in reproduction of amphioxus. Evidence in apparent favor of this conclusion is the fact that Chang et al. [2] stimulated sex steroidogenesis in *B. belcheri* by injecting mammalian gonadotropins, and Fang [13] claims to have stimulated spermiation in young amphibians by injecting them with homogenates of Hatschek's pit. Failure by Sahlin [8] to confirm in *B. lanceolatum* the presence of immunoreactive gonadotropin in Hatschek's pit, might be due to seasonal factors. Species of *Branchiostoma* breed seasonally [14, 15], so some seasonal variation in factors regulating reproduction might be anticipated. Species differences in the hormonal molecules might also play a role in producing these apparent differences.

If GnRH and gonadotropin are present in Hatschek's pit in amphioxi, and if they have a gonad-stimulating action, as Chang et al. [1, 2] and Fang [7, 13] claim, and as partly confirmed by us, then it would appear that the protochordates had evolved a form of vertebrate-like hormonal reproductive control long before evolution of the earliest vertebrates. Amphioxus is probably a degenerate form of a more complex protochordate ancestor [16–19], and the ascidians, likewise have evolved in a specialized direction from an ancestral form. The apparent preservation of a vertebrate-like reproductive regulatory mechanism in modern amphioxi, despite their apparent degeneracy, would seem to indicate that in earlier cephalochordates, such a mechanism may have been better developed. The fact that Hatschek's pit is open and exposed to the environmental water should make it an appropriate organ for sampling environmental thermal or chemical (pheromonal) factors that could seasonally stimulate gonadal activity. The neural gland of ascidians also retains a duct that extends directly into the stream of environmental water entering the pharynx, and therefore could involve an analogous system. In some carefully done experiments, Ruppert [20] has shown that the ciliated duct of *Ascidia interrupta* maintains a continues inward flow of water into the neural gland.

If it were based only on the immunocytochemical evidence that we have summarized here a thesis that depicts the evolution of the adenohypophysis from a chemoreceptive or olfactory structures would appear to be relatively tentative. However, some supportive evidence is available from the development of the vertebrate adenohypophysis, and also from the association of GnRH with the olfactory system. In embryos of lampreys, hagfish

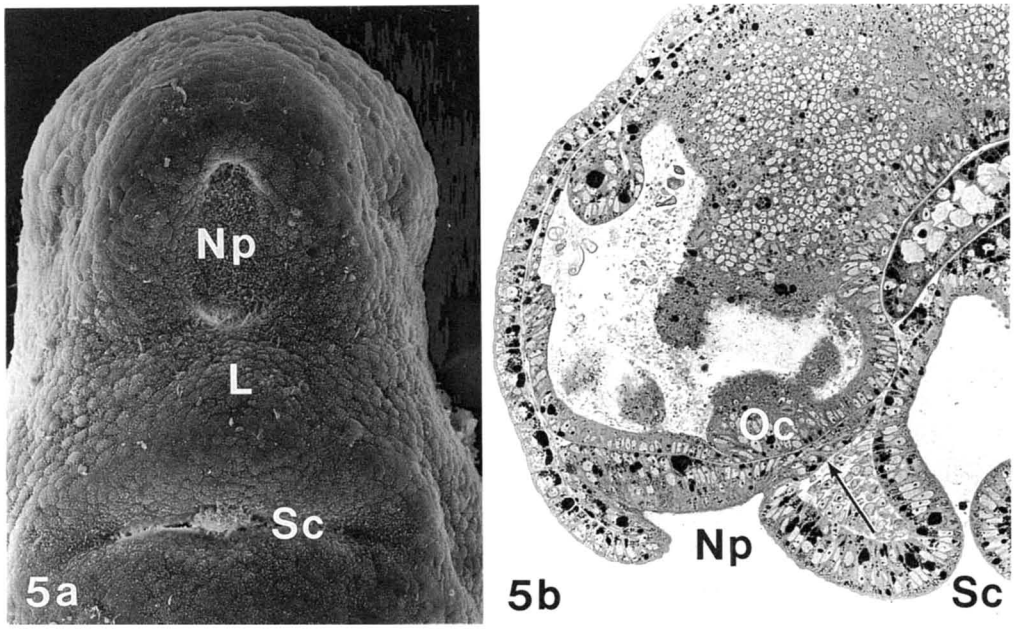


FIG. 5. **a**, Scanning electron micrograph; ventral view of the head of a recently hatched larval lamprey, *Lampetra japonica,* showing the stomodeal cleft (Sc) and nasopharyngeal opening (Np). The ciliated cells visible through the Np are the anlage of the olfactory organ; the adenohypophyseal anlage extends posteriorly from the olfactory epithelium and is hidden by an overlying fold of lip (L) tissue. **b**, Sagittal section of the head of a larval lamprey, *Lampetra japonica* of about the same stage as in **a**. The olfactory placode is the group of very tall cells immediately above the label Np (nasopharyngeal opening). Extending posteriorly from it and contiguous with it at the base, is the wedge-shaped adenohypophyseal anlage (arrow). Oc, optic chiasma. **a** and **b**, $\times 90$. **a** and **b**, Reproduced with permission from Honma, Chiba and Welsch [23].

and elasmobranchs the anlage of the adenohypophysis is immediately contiguous with the olfactory placode, and it is part of the same epithelial layer [21–23] (Fig. 5).

There is now a considerable literature describing the presence of immunoreactive GnRH in vertebrate embryos, as well as in adults, in the olfactory epithelium (placode), olfactory organ, olfactory tract, terminal nerves and in axons projecting from these to the hypothalamus of mammals, birds, amphibians and fishes [24–32]. Accordingly, evolution of a functional relationship between olfaction and reproduction has been an early and likely possibility.

If this is a primitive form of endocrine control over reproduction via an organ that directly samples pertinent environmental cues, then evolution of the more complex sense organ-hypothalamus-hypophysis form of reproductive (and other) regulation can be seen as a logical further step (Fig. 6). It is of interest that the evolved vertebrate adenohypophysis which is closed off from environmental contact and from sampling environmental changes, still retains direct secretory sensitivity to osmotic changes. Even *in vitro* cultured pituitary cells respond to changes in tonicity of culture medium by changes in secretion of prolactin [33, 34]. Furthermore, Olsson [6] has proposed that prolactin cells lining and near the open duct that connects the pars distalis to the mouth in certain adult fishes, such cells may be directly responsive to environmental salinity changes in regulating prolactin secretion. Here the analogy to Hatchek's pit and the ascidian neural gland is obvious.

In considering the adaptational features that would be advantageous in the regulation of reproductive function, it is obvious that synchrony with seasonal environmental phenomena is highly important. Synchrony of reproductive capacity of individuals within a population also is a basic

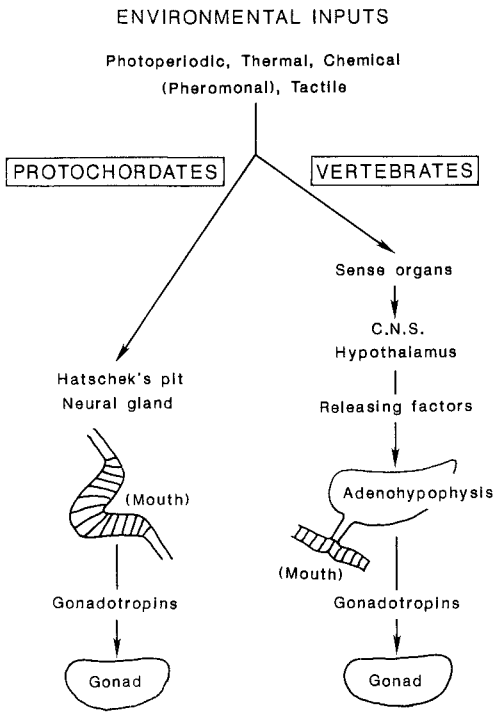


FIG. 6. Proposed evolutionary scheme from tropic function of the adenohypophysis.

adaptive need. Accordingly, a variety of systems have evolved in animal species to serve the purpose of linking reproduction to a seasonal environmental cue like photoperiod or temperature. Kanatani [35], for example, has explored a system in echinoderms of integumentary cells ("supporting cells"), which secrete a gonad-stimulating hormone and regulate spawning. The surface location of these cells indicates a probable sensitivity to environmental factors. Hatschek's pit of amphioxi, open to movement of environmental water in the mouth space, is clearly in a position appropriate for sensing pheromonal or thermal changes. Because of the small size and transparency of amphioxus, it could be photo-sensitive as well. The developing vertebrate adenohypophysis is also, at first, a structure in the mouth epithelium, a fact that has invited speculations concerning its homology with the similarly situated protochordate structures such as Hatschek's pit and the ascidian neural gland.

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